

Received 19 October; accepted 5 November 1999.

- Goate, A. *et al.* Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349, 704–705 (1991).
- Murrell, J., Farlow, M., Ghetti, B. & Benson, M. D. A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* 254, 97–99 (1991).
- Mullan, M. *et al.* A pathogenic mutation for probably Alzheimer's disease in the APP gene at the N-terminus of beta amyloid. *Nature Genet.* 1, 345–347 (1992).
- Cai, X. D., Golde, T. E. & Younkin, S. G. Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. *Science* 259, 514–516 (1993).
- Citron, M. *et al.* Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature* 360, 372–374 (1992).
- Suzuki, N. *et al.* An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. *Science* 264, 1336–1340 (1994).
- Selkoe, D. J. The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends Cell Biol.* 8, 447–453 (1998).
- Glennner, G. G. & Wong, C. W. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloidogenic derivative. *Science* 255, 728–730 (1984).
- Ladror, U. S., Snyder, S. W., Wang, G. T., Holzman, T. F. & Kraft, G. A. Cleavage at the amino and carboxyl termini of Alzheimer's amyloid-beta by cathepsin D. *J. Biol. Chem.* 269, 18422–18428 (1994).
- Dreyer, R. N. *et al.* Processing of the pre-beta-amyloid protein by cathepsin D is enhanced by a familial Alzheimer's disease mutation. *Eur. J. Biochem.* 224, 265–271 (1994).
- Saif, P. *et al.* Amyloidogenic processing of human amyloid precursor protein in hippocampal neurons devoid of cathepsin D. *J. Biol. Chem.* 271, 27241–27244 (1996).
- Tatnell, P. J. *et al.* Napsins: new human aspartic proteinases. Distinction between two closely related genes. *FEBS Lett.* 441, 43–48 (1994).
- Neill, D., Hughes, D., Edvardsson, B. K., Rima, B. K. & Allsop, D. Human IMR-32 neuroblastoma cells as a model cell line in Alzheimer's Disease research. *J. Neurosci. Res.* 39, 482–493 (1994).
- Asami-Odaka, A., Ishibashi, Y., Kikuchi, T., Kitada, C. & Suzuki, N. Long amyloid β -protein secreted from wild-type human neuroblastoma IMR-32 cells. *Biochemistry* 34, 10272–10278 (1995).
- De Strooper, B. *et al.* Deficiency of presenilin 1 inhibits cleavage of amyloid precursor protein. *Nature* 391, 387–390 (1998).
- Naruse, S. *et al.* Effects of PS1 deficiency on membrane protein trafficking in neurons. *Neuron* 21, 1213–1221 (1998).
- Vassar, R. *et al.* β -Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* 286, 735–741 (1999).
- Hussain, I. *et al.* Identification of a novel aspartic protease (Asp2) as β -secretase. *Molec. Cell. Neurosci.* [online] (<http://www.apnet.com/www/journal/cn/mcne.1999.0811>)
- Pirritola, T. *et al.* Longitudinal study of cerebrospinal fluid amyloid proteins and apolipoprotein E in patients with probably Alzheimer's disease. *Neurosci. Lett.* 249, 21–24 (1998).

Acknowledgements

We thank C. Himes, M. Fairbanks, J. Leone, T. Emmons, R. Drong, J. Slightom, G. Winterrowd and D. McKinley for their help, and J. McCall for his unflagging support and good humour.

Correspondence and requests for materials should be addressed to R.Y. (e-mail: riqiang.yan@am.pnu.com) or M.E.G. (e-mail: mark.e.gurney@am.pnu.com). Sequences are deposited in GenBank under the following accession numbers: human Asp1, AF200342; human Asp2, AF200343; mouse Asp2, AF200346; human Asp3, AF200343; and human Asp4, AF200345.

Purification and cloning of amyloid precursor protein β -secretase from human brain

Sukanto Sinha, John P. Anderson, Robin Barbour, Guriqbal S. Basl, Russell Caccavello, David Davis, Minhtam Doan, Harry F. Dovey, Normand Frigon, Jin Hong, Kirsten Jacobson-Croak, Nancy Jewett, Pamela Keim, Jeroen Knops, Ivan Lieberburg, Michael Power, Hua Tan, Gwen Tatsuno, Jay Tung, Dale Schenk, Peter Seubert, Susanna M. Suomensaar, Shuwen Wang, Donald Walker, Jun Zhao, Lisa McConlogue & Varghese John

Elan Pharmaceuticals, 800 Gateway Boulevard, South San Francisco, California 94080, USA

Proteolytic processing of the amyloid precursor protein (APP) generates amyloid β (A β) peptide, which is thought to be causal for the pathology and subsequent cognitive decline in Alzheimer's disease. Cleavage by β -secretase at the amino terminus of the A β peptide sequence, between residues 671 and 672 of APP,

leads to the generation and extracellular release of β -cleaved soluble APP¹, and a corresponding cell-associated carboxy-terminal fragment. Cleavage of the C-terminal fragment by γ -secretase(s) leads to the formation of A β . The pathogenic mutation K670M671 \rightarrow N670L671 at the β -secretase cleavage site in APP², which was discovered in a Swedish family with familial Alzheimer's disease, leads to increased β -secretase cleavage of the mutant substrate³. Here we describe a membrane-bound enzyme activity that cleaves full-length APP at the β -secretase cleavage site, and find it to be the predominant β -cleavage activity in human brain. We have purified this enzyme activity to homogeneity from human brain using a new substrate analogue inhibitor of the enzyme activity, and show that the purified enzyme has all the properties predicted for β -secretase. Cloning and expression of the enzyme reveals that human brain β -secretase is a new membrane-bound aspartic proteinase.

β -cleaved soluble APP (β -sAPP) was detected in membranes isolated from 293 cells stably overexpressing the 'Swedish' mutation, SweAPP751, by western blot analyses using the β -cleaved soluble APP (β -sAPP)-specific antibody Sw192 (ref. 4). Incubation of the membranes at pH 5.5 led to an increase in the cell-associated β -sAPP, and the appearance of a faster migrating species (Fig. 1). Treatment with O-glycanase resulted in the co-migration of both immunoreactive bands at the size of the lower band, which indicated that the smaller species resulted from β -cleavage of membrane-associated N-glycosylated immature APP (data not shown). These results are consistent with the specific cleavage of the full-length APP at the β -cleavage site by a membrane-bound proteinase activity. The membrane-bound β -cleavage activity exhibited a preference for acidic pH, with an optimum value of pH 5.5. Co-incubation with class-specific protease inhibitors, such as pepstatin, E-64 or phenylmethylsulphonyl fluoride, did not affect the generation of the β -cleaved APP (data not shown). Washing the membranes with 0.1% saponin under hypotonic conditions did not lead to loss of the membrane-associated β -cleavage enzyme activity (Fig. 1); therefore, we extracted P2 membranes⁵ in 0.1% Triton X-100, 0.1% Brij-35 or 0.1% β -octylglucoside to test the solubility of enzyme activity. The soluble supernatant fractions were assayed for β -cleavage activity, on an exogenous recombinant substrate, MBPC125Swe. Specific β -cleavage was detected only in the Triton X-100 extracts.

We analysed various tissues and cell lines for β -cleavage activity, by extracting P2 membranes from each source with 0.2% Triton X-100 and assaying for β -cleavage (Fig. 2a). Human and mouse brain, and brain regions had uniformly high levels of enzyme activity, whereas little activity was detected in other tissues. In different cell lines, neurons had the highest level of enzyme activity, whereas 293, Cos and Chinese hamster ovary (CHO) cells had lower levels. Cells

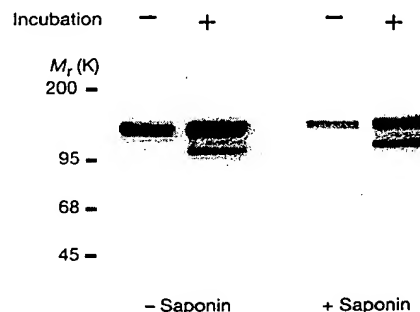


Figure 1 Endogenous substrate cleavage by β -secretase in P2 membranes. Membranes were prepared from 293 cells stably transfected with APP751, and either extracted with 0.1% saponin (+ saponin) or used directly (– saponin). Membranes in 0.1 M sodium acetate, pH 5.5 and 2% DMSO were either incubated (+) or solubilized without incubation (–). Samples were analysed by immunoblotting with the β -cleavage-specific 192sw antibody. M_r , relative molecular mass.

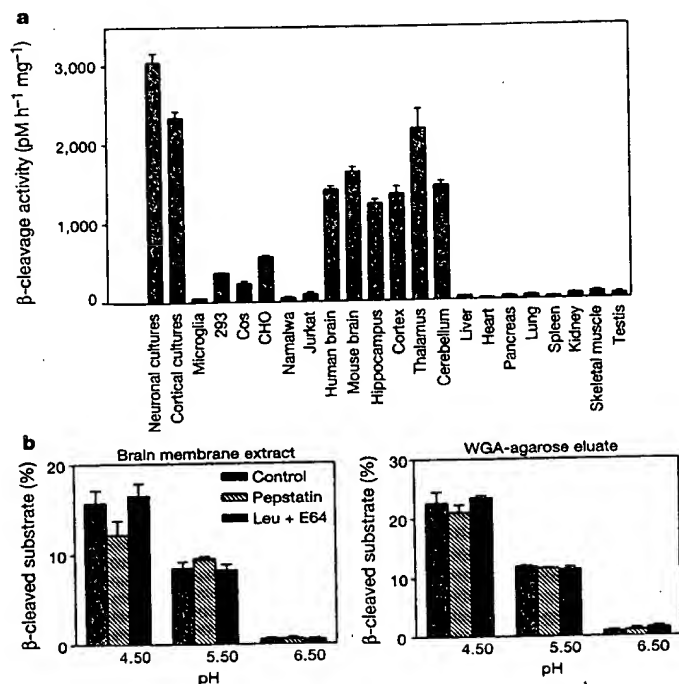


Figure 2 β-secretase activity in tissues and cell lines. **a**, Triton extracts of P2 membranes from the indicated cell types (first five samples) or tissues were assayed for β-secretase activity. **b**, Triton extracts of human brain membranes were assayed for β-secretase directly or after purification on WGA-agarose. Assays were performed at the indicated pH in the absence or presence of pepstatin or leupeptin and E-64 (Leu + E64).

of monocytic or lymphocytic origin did not have detectable enzyme activity. Thus, the enzyme activity is highest in cells of central nervous system (CNS) lineage, and found in cell lines commonly used for analysis of APP metabolism, in line with the observation that β-sAPP production is enhanced in CNS-derived cells, such as foetal neurons in culture¹.

An assay of solubilized extracts of human brain membranes showed a strong pH-dependent β-cleavage activity, which, like the activity in the 293Swe membranes, was not inhibited by pepstatin, leupeptin or E-64 (Fig. 2b). The soluble enzyme activity quantitatively bound to and eluted from wheat germ agglutinin (WGA) lectin-agarose. The pH dependence and inhibitor resistance remained unchanged through this (Fig. 2b) and all subsequent purification steps, and the enzyme activity migrated as a single species when analysed by size-exclusion chromatography (data not

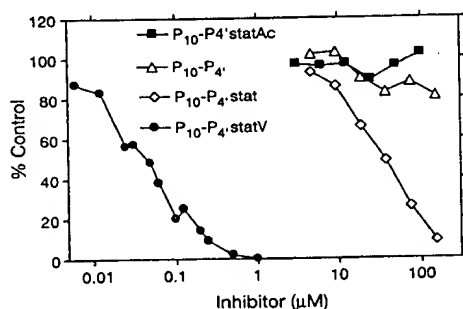


Figure 3 Inhibition of β-secretase by substrate analogue peptides. P₁₀-P₄': Thr662-Phe674 of APP751Swe; stat: statine substituted for P₁ leucine; statAc: substituted statine acetyl ester; statV: substituted statine and valine at P₁. Activity of β-secretase was assayed as described in Methods, except that peptides were added in DMSO stocks with a final concentration of DMSO of 2%. Values are plotted as percentages of DMSO control.

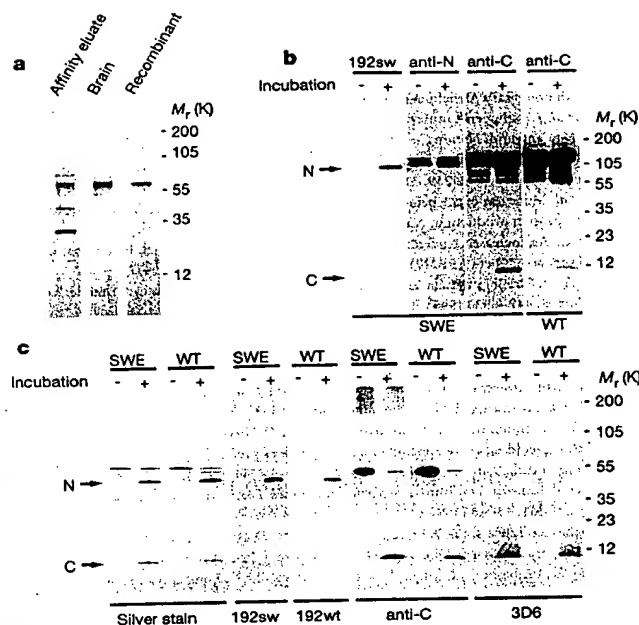


Figure 4 Purification and *in vitro* specificity of β-secretase. **a**, Silver-stained SDS-PAGE gels of β-secretase as eluted from a P₁₀-P₄' StatVal affinity column (affinity) and as finally purified from brain (brain) and β-secretase-transfected 293T cells (recombinant). **b**, Purified full-length APP, wild-type (WT) or Swedish (SWE) and partially purified β-secretase were either incubated (+) or analysed directly (-). Reactions were analysed by immunoblotting: anti-N, antibody 10F1; anti-C, antibody 13G8. Arrows indicate the positions of the N- and C-terminal fragments. **c**, Partially purified β-secretase was incubated with MBP-C125, WT or SWE variants, and analysed by silver-stained SDS-PAGE gels or immunoblotting.

shown), indicating that a single enzyme may be responsible for the human brain β-cleavage activity. In addition to the lack of inhibition by E-64 (cysteine proteinase inhibitor) and leupeptin (serine/cysteine proteinase inhibitor), no inhibition was seen with 3,4-DCIC (serine proteinase inhibitor), iodoacetamide (cysteine proteinase inhibitor) and 1,10-phenanthroline (metalloproteinase inhibitor). Although pepstatin was not inhibitory, even at 50 μM, some aspartic proteinases are insensitive to pepstatin⁶, and together with the acidic pH preference exhibited by the enzyme, it seemed probable that the β-cleavage was being carried out by a pepstatin-insensitive aspartic proteinase activity.

On the basis of this proposition, a P₁₀-P₄' (ref. 7), P₁ (S)-statine substituted substrate analogue was synthesized. The (S)-statine P₁₀-P₄' analogue dose-dependently inhibited the soluble β-cleavage activity with half-maximal inhibitory concentration (IC₅₀) ~40 μM, whereas the unmodified peptide (KTEEISEVNLDAEF) was not inhibitory (Fig. 3). Acetylation of the statine hydroxyl, or replacement of the S- with the R-statine enantiomer (data not shown), led to loss of inhibitory activity, indicating that, as in other statine-containing inhibitors for aspartic proteinases⁸, inhibitory potency is dependent on an unmodified hydroxyl residue in the appropriate configuration. Further improvement in the potency was achieved by substituting valine for aspartic acid at the P₁' position, which led to an inhibitor, P₁₀-P₄' StatVal, with IC₅₀ ~30 nM (Fig. 3).

Purification of the β-secretase enzyme activity from human brain was achieved by a sequential four-step procedure, incorporating an affinity-purification step with immobilized P₁₀-P₄' StatVal inhibitor peptide (Fig. 4a, affinity-eluate). The enzyme activity is purified ~300,000-fold using this procedure, yielding a ~70K (relative molecular mass, 70,000) protein homogeneous by silver-stained SDS polyacrylamide gel electrophoresis (PAGE) analysis (Fig. 4a, brain).

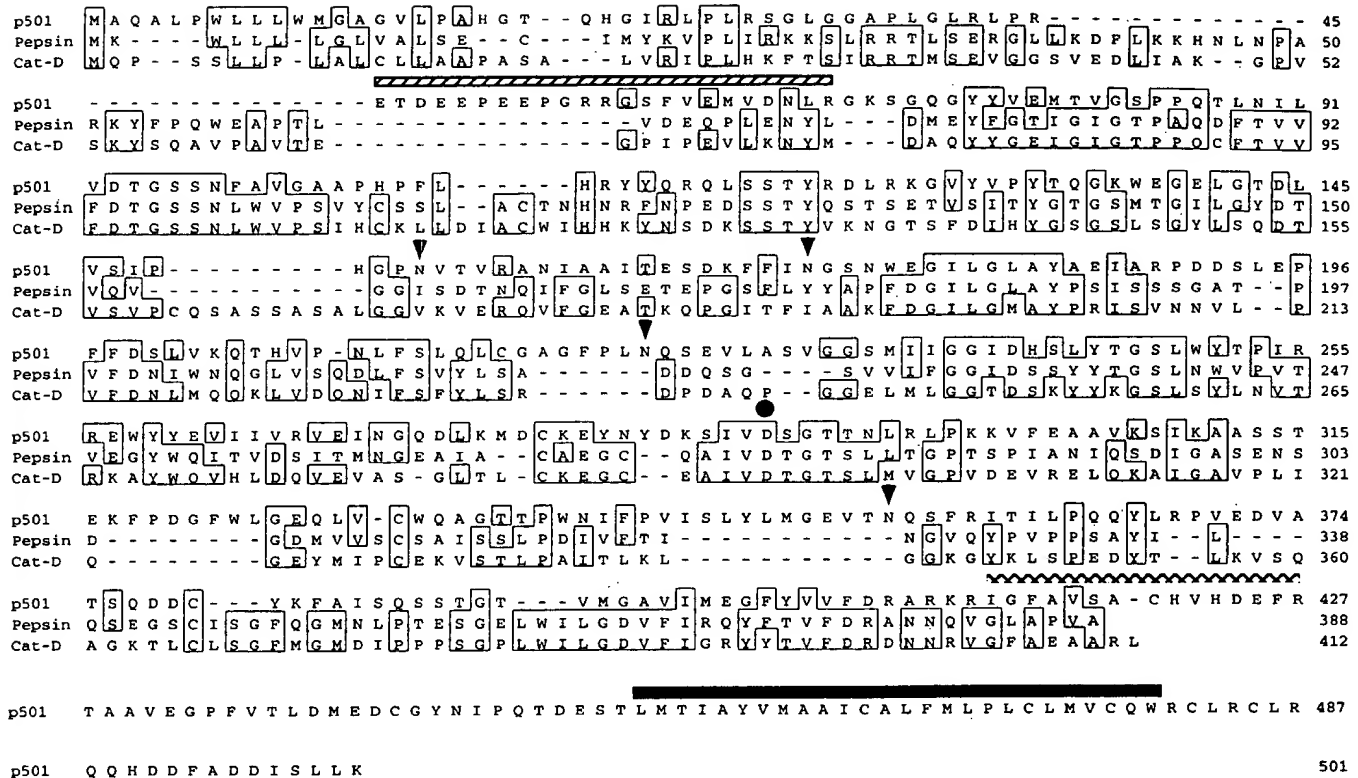


Figure 5 Sequence of β -secretase and homology with known aspartyl proteinases. The predicted amino-acid sequence of β -secretase (p501) is aligned with two well-characterized members of the human aspartyl proteinase family, pepsinogen and cathepsin D. Boxes represent amino acids that are identical in all three sequences. Hatched bar, the N terminus of mature p501, determined by N-terminal sequencing of the

purified protein. Cross-hatched bar, an internal amino-acid sequence from the purified protein determined from a peptidic fragment. Filled bar, transmembrane domain in p501, predicted by hydropathy analysis of the sequence. Filled circles, active-site aspartic acid residues. Arrowheads indicate the four predicted N-linked glycosylation sites in the p501 sequence.

The purified enzyme cleaved both MBPC125Swe and wild-type substrates specifically at their β -cleavage sites (Fig. 4c, silver stain, anti-C), generating N-terminal fragments that were immunoreactive with the β -sAPP neo-epitope-specific Sw192 and Wt192 (ref. 1) antibodies (Fig. 4c, 192sw, 192wt). The corresponding C-terminal fragments (Fig. 4c, 3D6), which reacted with 3D6 (ref. 9), monoclonal antibody specific to the free N terminus of A β 1-5, were also detected, providing evidence of a single endopeptidic cleavage. The cleavage sites were confirmed by N-terminal sequence analyses. Incubation of the brain enzyme with purified full-length APPSwe also resulted in specific cleavage at the β -site, as determined by the generation of single N- and C-terminal fragments (Fig. 4b).

Edman degradation of purified human brain β -secretase revealed a single N-terminal sequence, ETDEEPEEPGRGSGFVEMVDNLR, which we used for isolation of complementary DNA clones encoding full-length β -secretase by a combination of polymerase chain reaction (PCR) and conventional cDNA library screening. Sequence analysis of cDNA clones isolated from human neurons and human foetal brain identified open reading frames that were predicted to encode a polypeptide comprising 501 amino acids, p501. This sequence is identical to a recently described β -secretase clone¹⁰, which was obtained from a 293 cell library using expression cloning. The N-terminal sequence obtained from the purified human brain enzyme corresponded to residues 46–66 of the predicted protein sequence (Fig. 5), indicating that the mature enzyme is generated from a preproenzyme.

Co-transfection of p501 cDNA with either WtAPP751 or SweAPP751 in 293T cells led to marked increases in β -sAPP production (Fig. 6b) and substantial increases in A β peptide (Fig. 6a). The large increase in β -sAPP is accompanied by a corresponding

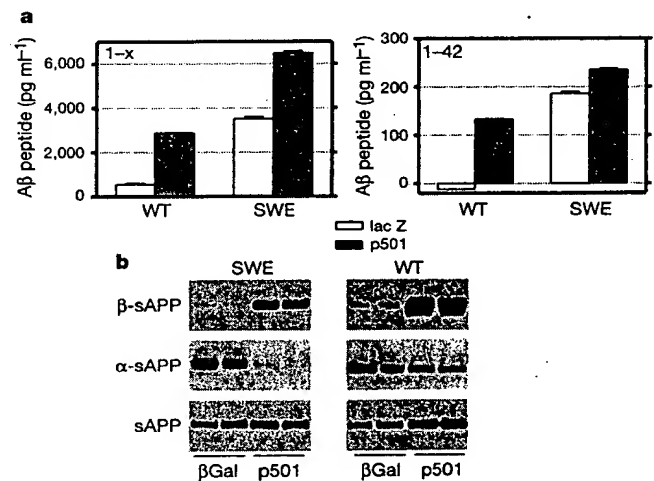


Figure 6 Transfection of cells with β -secretase. Cells were co-transfected with either wild-type (WT) or Swedish (SWE) APP and either the β -secretase clone p501 or β -galactosidase control. Co-expression of β -gal or p501 and APP coding sequences was directed from the CMV promoter peptide in vectors that did not permit replication in 293T cells. **a**, Media were analysed for A β peptide using ELISAs specific for all A β C termini (1–x) or A β 42 (1–42). **b**, Media were analysed by immunoblotting to measure secreted APP (sAPP): α -sAPP (antibody 1736), β -sAPP (antibodies 192 or 192Swe) and total sAPP (antibody 8E5).

decrease in α -sAPP levels (Fig. 6b, α -sAPP), suggesting a very efficient cleavage of full-length APP at the β -cleavage site. Thus, p501 overexpression leads to increased β -secretase cleavage of both WtAPP and SweAPP, accompanied by increased concentrations of A β peptide.

The recombinant enzyme was purified using cation exchange and the inhibitor affinity matrix (Fig. 4a, recombinant), and its N-terminal sequence was determined. About 90% of the purified protein N-terminal sequence corresponded exactly to that determined for the purified brain enzyme (residue 46). About 10% of the total sequence started at residue 22, the proenzyme form generated upon removal of the signal peptide (residues 1–21). Expression of the recombinant enzyme is thus accompanied by efficient cellular proteolytic processing indistinguishable from that of the native enzyme purified from human brain.

Northern blot analyses (data not shown) showed high expression in brain and pancreas, as previously described¹⁰; however, whereas brain displayed high enzymatic activity (Fig. 2a), pancreas had very low activity. It may be that post-transcriptional regulation, possibly at the level of translation, regulates pancreas β -secretase activity.

The activity of human brain β -secretase, both *in vitro* and in cells, is highly specific for the β -cleavage site. Overexpression of the enzyme leads to marked increases in β -sAPP and A β peptide levels. The enzyme shows enhanced cleavage of Swedish as compared to wild-type substrate, in agreement with results obtained from cellular studies. The highest levels of enzyme activity are found in cells and tissues of CNS origin, which supports its proposed role as human brain β -secretase. The pH-activity profile of the enzyme is acidic, consistent with cellular studies of APP metabolism that intracellular acidic compartments are involved in β -, but not α -, secretase cleavage events⁴.

Our data show that activation of β -secretase from the proenzyme form occurs efficiently in cell lines. β -secretase enzyme activity levels correlate well with the propensity of the cell line or tissue to generate A β peptide from APP. As with HIV proteinase¹¹, and illustrated here by the peptide-based transition state analogue, potent and selective inhibitors of this enzyme can be generated. Further optimization with structure-based drug design should lead to the discovery of small molecules that would be effective in inhibiting A β peptide production in Alzheimer's disease. □

Methods

APP substrates

The 125 C-terminal residues of APP, in both the wild-type and the Swedish variants, were fused to the C terminus of MBP in the *Escherichia coli* expression vector pMALc (New England Biolabs). The fusion proteins were induced in bacterial cultures and purified by solubilization with 1% Triton X-100/7 M urea, followed by chromatography on amylose-agarose. The bound proteins were eluted with 10 mM maltose in 20 mM Tris, pH 7.5, 1% Triton X-100, 0.1 M NaCl, diluted 1:1 with 6 M guanidine, and stored in frozen aliquots (0.1 mg ml⁻¹ fusion protein in 10 mM Tris, pH 7.5, 0.2% Triton X-100, 0.15 M guanidine-HCl).

Full-length APP was prepared from 293 cells stably transfected with Swedish or wild-type APP. P2 membranes were washed with 20TE (20 mM Tris, 2 mM EDTA) with 0.5M NaCl and extracted with 20TE with 0.5% Triton X-100. Full-length APP was purified by anion exchange and immuno-affinity chromatography¹² on 13G8 coupled to NHS-Sephacrose.

β -secretase enzyme assay

β -cleavage assays were carried out in 20 mM sodium acetate, pH 4.8, 0.06% Triton X-100, with 10 μ g ml⁻¹ MBPAPP125. Reaction mixtures were incubated at 37 °C for 1–2 h, and the quenched reaction mixtures were then loaded onto 96-well plates coated with a polyclonal antibody raised to MBP. Generated β -cleaved product was detected using biotinylated Sw192 or biotinylated Wt192 as specific reporter antibodies and quantitated against the appropriate MBP-C26 standard.

Enzyme isolation

Crude membrane fractions were obtained from frozen human brain tissue. The membrane pellets were extracted with 20 mM MES, pH 6, 0.15 M NaCl, 0.5% Triton X-100. The soluble extract obtained by centrifugation was loaded onto WGA-agarose at pH 7.5, and the column eluted with 6% chitin hydrolysate after washing. We diluted the eluate fourfold into 20 mM sodium acetate, pH 5, adjusted the pH to 5.0, and passed it through a Hi-Trap SP column. The SP flow-through was adjusted to pH 4.5 with acetic acid and applied to a column of P₁₀-P₄ statV coupled to NHS-Sephacrose. The column was washed and eluted

with 0.2 M NaCl, 40 mM sodium borate, pH 9.5, and 2.0% Triton X-100. We removed contaminating proteins by anion exchange on MiniQ. N-terminal sequence analysis was carried out by Argo Bioanalytica.

Cloning and expression

Screening of the Origene human foetal brain Rapid-Screen cDNA Library Panel by degenerate PCR, using oligos 5'-GAGAGACGA(G/A)GA(G/A)CC(A/T)GAGGAGCC-3' and 5'-CGTCACAG(G/A)TT(G/A)TCTACCATCTC-3', identified a partial clone encoding the amino-acid sequence from the purified enzyme. Radiolabelled probe derived from this clone was used to screen a size-selected human foetal primary neuronal cell library generated in a mammalian expression vector to obtain the full-length clone, p501. Wild-type or Swedish APP751 was transiently co-transfected with either p501 or β -gal control into 293T cells (Edge Biosciences). Co-expression of β -gal or p501 and APP coding sequences was directed from the CMV promoter in vectors that did not permit replication in 293T cells. Medium was collected 48 h after transfection.

Antibodies

Antibodies were directed against the following portions of the APP751 sequence: 10F1, residues 20–304 (ref. 9); 13G8, 727–751; 8E5, 495–643 (ref. 9); 192wt and 192sw, specific for β -cleaved APP wild-type and Swedish variants, respectively¹⁴; 1746, specific for α -cleaved APP (courtesy of D. Selkoe); 3D6, A β 1–5, specific for the free N terminus⁹.

Received 1 November; accepted 8 November 1999.

- Seubert, P. *et al.* Secretion of beta-amyloid precursor protein cleaved at the amino terminus of the beta-amyloid peptide. *Nature* **361**, 260–263 (1993).
- Mullan, M. *et al.* A pathogenic mutation for probably Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. *Nature Genet.* **1**, 345–347 (1992).
- Citron, M. *et al.* Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein secretion. *Nature* **360**, 672–674 (1992).
- Knops, J. *et al.* Cell-type and amyloid protein-type specific inhibition of Ab release by bafilomycin A1, a selective inhibitor of vacuolar ATPases. *J. Biol. Chem.* **270**, 2419–2422 (1995).
- Valdes, F., Munoz, C., Faria-Velasco, A. & Orrego, F. Subcellular distribution of rat brain cortex high-affinity, sodium-dependent, glycine transport sites. *Brain Res.* **122**, 95–112 (1977).
- Narutaki, S., Dunn, B. M. & Oda, K. Subsite preference of pepstatin-insensitive carboxyl proteinases from bacteria. *J. Biochem. (Tokyo)* **125**, 75–81 (1999).
- Schechter, I. & Berger, A. On the size of the active site in proteinases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**, 157–162 (1967).
- Rich, D. H. in *Proteinase Inhibitors* (eds Barrett, A. J. & Salvesen, G.) (Elsevier Science, Amsterdam, 1986).
- Johnson-Wood, K. *et al.* Amyloid precursor protein processing and A β ₄₂ deposition in a transgenic mouse model of Alzheimer disease. *Proc. Natl Acad. Sci. USA* **94**, 1550–1555 (1997).
- Vassar, R. *et al.* β -secretase cleavage of the Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735–741 (1999).
- Lin, J. H., Ostovic, D. & Vacca, J. P. The integration of medicinal chemistry, drug metabolism, and pharmaceutical research and development. The story of Crivarin, an HIV proteinase inhibitor. *Pharm. Biotechnol.* **11**, 233–255 (1998).
- Knops, J. *et al.* Isolation of Baculovirus-derived secreted and full-length β -amyloid precursor protein. *J. Biol. Chem.* **266**, 7285–7290 (1991).

Correspondence and requests for materials should be addressed to S.S. (e-mail: ssinha@elanpharma.com). The nucleotide sequence of p501 has been deposited in the GenBank under accession number AF201468.

Axon routing across the midline controlled by the *Drosophila* Derailed receptor

Joshua L. Bonkowski*, Shingo Yoshikawa*, David D. O'Keefe, Audra L. Scully & John B. Thomas

Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, California 92186, USA

* These authors contributed equally to this work

In nervous systems with symmetry about the midline, many neurons project axons from one side to the other. Although several of the components controlling midline crossing have been identified^{1–4}, little is known about how axons choose the appropriate pathway when crossing. For example, in the *Drosophila* embryo axons cross the midline in one of two distinct tracts, the anterior or posterior commissure (AC or PC, respec-